*Bactericidal Properties Conferred on the Blood by Intravenous Injections of Diamino-Acridine Sulphate.**

By C. H. Browning and R. Gulbransen.

(Communicated by Prof. R. Muir, F.R.S. Received August 11, 1917.)

(From the Bland-Sutton Institute of Pathology, Middlesex Hospital.)

Attempts to achieve "internal disinfection," that is to say, to kill organisms in the body of infected animals by means of drugs, have hitherto afforded little promise of success in the case of the common pathogenic bacteria, the only exception being the action of ethyl-hydrocuprein in pneumococcus infections, discovered by Morgenroth with his co-workers, Levy and others. The reasons for the failure are probably to be attributed mainly to two facts; in the first place, antiseptics in general enter into combination with proteins of the tissues and body fluids, either by a process of physical adsorption or by the formation of chemical compounds. In either case, the usual result is that the bactericidal action of a substance, as determined in a watery medium, is greatly reduced by protein-solutions, *e.g.*, serum. Secondly, the majority of chemical antiseptics are general protoplasm poisons, and exert on mammalian tissues a degree of toxic action equal to, or greater than, that which they exhibit towards micro-organisms; hence these substances prove lethal for animals in doses which are insufficient to confer bactericidal properties on the body fluids.

The classical example of such failures was afforded by mercuric chloride, which Koch employed in the hope of treating effectively anthrax septicæmia in animals. Ehrlich and Bechhold investigated compounds which were more potent antiseptics than any hitherto known, among the most active being tetrabrom- (and chlor-) ortho-biphenol; they found, however, that the bactericidal properties of these substances were also greatly reduced when the organisms were suspended in a serum medium. Thus, tetrachlor-ortho-biphenol in a concentration of 1 : 320,000 prevented the growth of diphtheria bacilli in bouillon, whereas in serum growth occurred in the presence of 1 : 10,000 of this reagent. Similarly we have found that the dose of perchloride of mercury which is required to inhibit completely the growth of *Staphylococcus aureus* or *B. coli* in serum is 100 times greater than that which produces this effect in watery medium containing a small amount of nutrient peptone (0·7 per cent.).

* We are indebted to the Medical Research Committee for a grant towards the expenses of this work.

Browning and Gilmour, while investigating relationships between constitution and bactericidal action among basic benzol-derivatives, found that diamino-acridine was more powerfully bactericidal in the presence of serum than in ordinary peptone-water-agar medium. Subsequent observations by Browning, Gulbransen, Kennaway and Thornton have confirmed and extended this result; it has been found that a number of diamino-acridine derivatives with substituted methyl-groups either in the amino side-chains or in the benzol rings, or in both situations, *e.g.*, the dye acridine yellow, are all enhanced in their bactericidal action by serum. This is likewise the case with Benda's compound, 3 : 6-diamino-10-methyl-acridinium chloride.

In the presence of serum this group of substances constitutes the most potent bactericidal agents known, and the property of being enhanced in this activity by serum, so far as we are aware, is shared by no other type of chemical compound which has been investigated. On account of this property, together with the fact that they are comparatively non-toxic to mammalian tissues, and devoid of inhibitory effect on phagocytosis, diamino-acridine salts (sulphate and chloride) and diamino-methyl-acridinium chloride have been recommended as therapeutic substances for local application in the treatment of bacterial infection in wounds. Their bactericidal action is slowly progressive and the maximum effect is attained only after a considerable time, thus concentrations of these substances which at first merely inhibit proliferation of the organisms ultimately prove lethal; in this respect they differ from such substances as phenol, mercuric chloride, and sodium-toluene-para-sulphochloramide. Thus, if mixtures of serum with varying proportions of any of the latter compounds are inoculated with a suspension of a culture of living micro-organisms, such as *Staphylococcus aureus* or *B. coli*, and are placed in the incubator at 37° C., it is found on making subcultures at intervals, that if a given concentration of antiseptic has not proved lethal in two hours, its presence has little effect in preventing the occurrence of active multiplication of the organisms subsequently.

On the other hand, with the acridine compounds the effect in two hours is very slightly greater than with mercuric chloride, but the concentration which proves lethal in 24 hours is only a tenth to a twentieth of the lethal concentration of mercuric chloride. Thus, it was found that a concentration of 1 : 10,000 of mercuric chloride killed these organisms in serum in two hours, but with a concentration of 1 : 20,000 the bacteria were still alive after 24 hours and had multiplied actively. With diamino-methyl-acridinium chloride, however, the lethal concentration after two hours was 1 : 20,000, and after 24 hours a strength of 1 : 100,000 and 1 : 200,000 had killed *B. coli* and *Staphylococcus aureus* respectively; diamino-acridine sulphate is

similar in its action. The relative lack of toxic effect on leucocytes is shown by the fact that when a volume of human "leucocyte cream" along with a volume of human serum, the mixture containing 1:10,000 of the acridine derivatives, is incubated for two hours at 37° C., and staphylococci are subsequently added—time then being allowed for phagocytosis to occur—an estimation of the phagocytic count yields over 50 per cent. of the number of cocci ingested by leucocytes subjected to similar conditions except that the dye was omitted from the mixture; on the other hand, 1:10,000 of mercuric chloride reduces the phagocytic power to a much greater extent. Now in the case of the mercury salt in serum, this concentration represents practically the limit beyond which effective antiseptic action does not take place; on the other hand, the acridine compounds in 1:10,000 dilution in serum are powerfully antiseptic and ultimately prove lethal to the organisms.

Experiments have shown that of the two substances diamino-acridine sulphate* is the more suited for direct injection into the blood stream, as it is less toxic and has less agglutinating action on the red blood corpuscles than diamino-methyl-acridinium chloride. Accordingly, the present investigation was undertaken with a view to ascertaining whether it was possible to render the blood serum antiseptic without at the same time damaging the health of an animal treated in this fashion; the results have shown that this aim could be realised.

The following preliminary observations, the results of which are shown in the Table, served to determine the relative toxicity of diamino-acridine sulphate and diamino-methyl-acridinium chloride, and also their bactericidal power for *Staphylococcus aureus* and *B. coli*.

Substance.	Maximum non-lethal dose for a normal 20-grm. mouse.	Bactericidal concentration for			
		Staph. aureus in		*B. coli* (Escherich) in	
		0·7-per-cent. peptone water.	Serum.	0·7-per-cent. peptone water.	Serum.
	grm.				
Diamino-acridine sulphate...	0·003	1:20,000	1:200,000	1:4000	1:100,000
Diamino-methyl-acridinium chloride	0·0006	1:20,000	1:200,000	1:1300	1:100,000

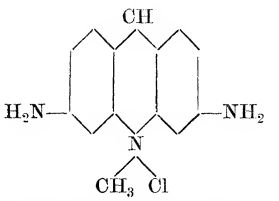
* We have pleasure in expressing our indebtedness to Drs. Barger and Ewins, of the Department of Biochemistry and Pharmacology of the Medical Research Committee, for their kindness in preparing for us the supply of this substance which we required.

Method of the Tests.—The toxicity for mice was determined by injecting watery solutions subcutaneously, the dose being so arranged that a 20-grm. mouse received a volume of 1 c.c.; to animals of other weights corresponding volumes were given, but mice not exceeding the limits of 15–25 grm. were selected for the tests.

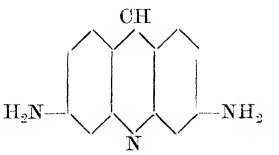
The bactericidal concentration was found thus: The substance to be tested, in a volume usually not exceeding 0·1 c.c., was added to small test-tubes containing 1 c.c. of the culture medium, which consisted in one series of 0·7 per cent. of peptone water, and in the other of undiluted serum, usually from the ox (previously heated at 56° C. for an hour), and then 0·1 c.c. of a 1 : 20,000 dilution in saline of a peptone water culture (previously incubated for 24 hours at 37° C.) was added. A control was made with peptone water or serum without antiseptic; one loopful of this mixture, when stroked immediately on agar, yielded about twenty colonies of *Staphylococcus* or *B. coli*. The tubes were then placed at 37° C., and were examined at the end of 24–48 hours, in order to determine the concentration of antiseptic which killed the organisms introduced; the development of turbidity, of course, indicated the occurrence of definite proliferation of the bacteria, but sub-cultures were made also on agar and in peptone water. The results of both methods of subculture corresponded in general, but it was sometimes found that cultures containing antiseptic which showed no turbidity after incubation, and in which, therefore, little or no multiplication of organisms had occurred, still contained living bacteria.

It is to be noted that we selected the quantity of bacteria employed for the inoculation dose because, when added to the standard volume of fluid used in our tests (1 c.c.), one loopful of the mixture yielded a convenient number of colonies (about twenty) for estimating subsequent increase or decrease. But the employment of 0·1 c.c. of undiluted culture, *i.e.* a 20,000-fold dose, required for sterilisation a concentration of antiseptic only $2\frac{1}{2}$–5 times greater than that recorded above.

Diamino-methyl-acridinium chloride was prepared by Benda for Ehrlich and was found to possess very marked curative properties for experimental trypanosome infections, hence the name "trypaflavin" was applied to it. The remarkable properties of this substance and other acridine compounds as bactericidal agents, so far as we know, was not suspected. It is of interest, in regard to the relationship existing between chemical constitution and therapeutic action, that the trypanocidal property depends greatly on the presence of the methyl-group attached to the nitrogen atom. This is implied, though not expressly stated in Benda's work.



Diamino-methyl-acridinium chloride.



Diamino-acridine (base).

We have investigated the question in the case of mice infected with *Tr. rhodesiense.** The mice were inoculated by subcutaneous injection of a dilution of richly infected blood. After several days, when parasites were readily found in their blood, a series of animals received varying doses of diamino-methyl-acridinium chloride and diamino-acridine sulphate. The maximum dose of the acridinium compound tolerated by infected animals (0·0003 grm.) caused disappearance of the parasites from the blood for a number of days as a rule, so that great protraction of the infection resulted. With diamino-acridine sulphate, although a much larger dose was well tolerated (0·0015 grm.), there was, as a result, only very slight protraction of the infection as compared with the untreated controls. The latter invariably died, although the number of trypanosomes in the blood, after increasing to a maximum, frequently receded spontaneously, only to increase again prior to death. Thus, although the infection with this strain of trypanosomes in mice was by no means ideal for chemo-therapeutic observations, the fact of the great superiority of the acridinium compound in this respect was clearly apparent.

The main experiment consisted in introducing diamino-acridine sulphate in 0·85-per-cent. NaCl solution intravenously into rabbits, and then withdrawing specimens of blood at intervals. The blood was allowed to coagulate, and the serum was withdrawn under aseptic precautions, and was freed from cellular elements by centrifugalising. Quantities of 1 c.c. each of unheated serum were then inoculated with 0·1 c.c. of a 1 : 20,000 dilution of a 24-hour peptone water culture of *Staphylococcus aureus* or *B. coli,* and these cultures were incubated at 37° C. for 48 hours or longer. By way of control, a specimen of blood was taken before the injection, and was similarly inoculated. The majority of the animals manifested no signs of illness during or after the injections, and were alive and well many weeks later. The following are characteristic examples :—

Rabbit No. I (weight, 1950 grm.).—0·13 grm., dissolved in 45·5 c.c. of 0·85-per-cent. NaCl solution, injected into the auricular vein in the course of 9½ minutes (dose = 0·066 grm. per kilogramme of body weight).

* We are indebted to Prof. Warrington Yorke for the strain.

A. Serum of blood taken before injection, inoculated with (*a*) *Staphylococcus aureus*, (*b*) *B. coli*; both gave abundant growth (marked turbidity after 24 hours at 37° C.).

B. Serum of blood withdrawn five minutes after the injection—

(i) Undiluted: remained perfectly clear after inoculation, in the case of both organisms, and subcultures on agar showed no growth;

(ii) A mixture of 50 per cent. serum B + 50 per cent. serum A gave no growth of either organism after inoculation;

(iii) A mixture of 25 per cent. serum B + 75 per cent. serum A yielded a growth of *Staphylococcus*, but not of *B. coli*, after inoculation.

Rabbit No. II (weight, 1850 grm.).—0·13 grm. dissolved in 40 c.c. of 0·85-per-cent. NaCl solution injected into the auricular vein in the course of 4½ minutes (dose = 0·07 grm. per kilogramme body weight).

A. Serum of blood taken before injection, inoculated with (*a*) *Staphylococcus aureus*, (*b*) *B. coli*, gave abundant growth after 24 and 48 hours at 37° C. respectively.

B. Serum of blood withdrawn 15–25 minutes after the injection—

(1) Undiluted: gave no growth after inoculation with either organism.

(2) Diluted with an equal volume of serum A: grew *Staphylococcus* but not *B. coli* after inoculation.

(3) Twenty-five per cent. serum B + 75 per cent. serum A gave a growth of *B. coli* after inoculation.

C. Serum of blood withdrawn 2½ hours after the injection—

(1) Undiluted: grew *Staphylococcus*, but not *B. coli* after inoculation.

(2) A 50 per cent. dilution with specimen A grew *B. coli* also.

Rabbit No. III (weight, 1420 grm.).—0·07 grm., dissolved in 20 c.c. of 0·85-per-cent. NaCl solution, injected into the auricular vein in the course of six minutes (dose = 0·05 grm. per kilogramme body weight).

A. Serum withdrawn before injection, inoculated with (*a*) *Staphylococcus*, (*b*) *B. coli*, gave abundant growths after 24 hours at 37° C. (turbid); the addition of 1 : 100,000 diamino-acridine sulphate to the serum *in vitro* prevented growth in the case of both organisms.

B. Serum withdrawn two hours after injection, when inoculated with *Staphylococcus aureus* and *B. coli*, remained perfectly clear after three days' incubation at 37° C., thus showing that little or no multiplication of the inoculated organisms had taken place, but subculture on agar yielded a few colonies in each case.

In the experiments quoted above fresh unheated serum was employed, and it might be inferred that the natural bactericidal property of serum, to which

Nuttall first drew attention, had contributed in a considerable measure to the results obtained; but it is to be noted that in all cases the controls, consisting of fresh serum obtained immediately before the injection, yielded after inoculation abundant growths of both organisms employed. Thus there is evidence in our experiments that the natural bactericidal property of the serum was not a decisive factor in producing the antiseptic effect. *Staphylococcus* is not killed by serum, as Wright and Windsor pointed out in the case of the human subject. In the case of *B. coli*, serum from the rabbit causes a phase of bactericidal action, which is frequently succeeded by multiplication (Chick); this latter phase of multiplication was of constant occurrence in our investigations.

In order to demonstrate further that the bactericidal effect following the injection of diamino-acridine sulphate was independent of properties of the fresh serum, experiments were also carried out with heated serum.

Example.—Rabbit (weight, 1510 grm.): 0·09 grm. diamino-acridine sulphate, dissolved in 20 c.c. of 0·85-per-cent. NaCl solution, injected into the auricular vein in the course of five minutes (dose = 0·06 grm. per kilogramme of body weight). Specimens of serum (*a*) fresh and (*b*) after heating for one hour at 56° C.—taken (A) before, (B) 17 minutes after, the injection—were inoculated with *Staphylococcus aureus* and *B. coli* as described above.

The results after incubation for 72 hours at 37° C. were as follows:—

Inoculation with *Staph. aureus.*				
Serum.	A (before injection) undiluted	B (after injection) undiluted	B 75 per cent. + A 25 per cent.	B 25 per cent. + A 75 per cent.
Fresh	Abundant growth (turbid)	No growth* (clear)	No growth* (clear)	Growth (turbid)
Heated one hour at 56° C.	Abundant growth (turbid)	No growth* (clear)	No growth* (clear)	Growth (turbid)
Inoculation with *B. coli.*				
Serum.	A undiluted	B undiluted	B 75 per cent. + A 25 per cent.	B 25 per cent. + A 75 per cent.
Fresh	Abundant growth† (turbid)	No growth* (clear)	No growth (clear)	No growth* (clear)
Heated one hour at 56° C.	Abundant growth† (turbid)	No growth* (clear)	No growth (clear)	Growth

* Subcultures on agar yielded scanty colonies.

† With *B. coli* growth in the heated serum was abundant after 24 hours; in the unheated serum the growth was scanty after 24 hours, but became marked later; this may be taken as evidence of the natural inhibitory effect of fresh serum.

Thus, it has been shown that in rabbits, a dose of diamino-acridine sulphate, which is well tolerated when introduced directly in the blood stream, is capable of rendering the blood serum antiseptic or of augmenting greatly its bactericidal power, and that this property is still manifested several hours after the injection. A great part of the substance rapidly enters the muscles, which become of a distinct yellow tint, but this does not affect the fact just stated. Accordingly, there appears to be here a very promising indication as to the lines on which a chemo-therapeutic agent applicable to cases of bacterial septicæmia is to be sought.*

Diamino-acridine sulphate is absorbed from the alimentary tract, and, after administration by this route or intravenously, the urine soon exhibits the canary-yellow fluorescence, best seen on dilution, which is so characteristic of weak solutions of the acridine compounds. There is also excretion of the substance by the bile. Observations on the human subject treated by

* We are indebted to Dr. H. H. Dale, F.R.S., for the blood-pressure record of an experiment in which he injected 0·3 grm. of the diamino-acridine sulphate in a volume of 100 c.c. intravenously into a monkey weighing 4·3 kgrm., under an anæsthetic. Specimens of serum taken during and after the injection (see below), both fresh, and also after heating for half an hour at 56° C., failed to yield growths after inoculation with staphylococcus and *B. coli*.

The results are as follows :—

Time.	Anæsthetic.	Blood-pressure at end of period.	Total amount of substance injected (1 : 300 solution) in each period.
Commencement of record	A.C.E.	100 mm. Hg	0
17 minutes later	,,	90 ,, ,,	0
16 ,, ,, ...	,,	75 ,, ,,	22 c.c. run in intermittently.
18 ,, ,, ...	,,	Rapid fall towards the end of this period to 45 mm., and heart irregularly inhibited	40 c.c. run in continuously (at first 2 c.c. per minute, soon increased to 3 c.c. per minute)
15 ,, ,, ...	Ether	55 mm. Hg	0 (20 c.c. blood withdrawn from carotid)
5 ,, ,, ...	,,	60 ,, ,,	0
13 ,, ,, ...	,,	60 mm. Hg (heart rather severely inhibited)	38 c.c. (20 c.c. blood withdrawn from carotid)
21 ,, ,, ...	,,	60 mm. Hg (heart inhibition passed off)	0 (20 c.c. blood withdrawn, then animal bled completely)

The conclusion from this single experiment is that when an animal of similar susceptibility receives an intravenous injection under an anæsthetic, administration at as great a rate as 0·0025 grm. per minute per kilogramme of body weight causes some danger to the heart (under similar conditions this would mean that an average man weighing 60 kgrm. should not receive an intravenous injection at a rate exceeding 0·15 grm. per minute or 50 c.c. of a 1 : 330 solution). There is, of course, considerable likelihood that the anæsthetic tends to increase the susceptibility of the heart.

intravenous injections of diamino-methyl-acridinium chloride have shown that, after a dose of 0·15–0·3 grm. (in the form of a 1 : 1000 solution in physiological saline), fully a third of this amount can be accounted for in the urine passed during the subsequent two days.*

Thus it is possible that these or allied substances may prove of value in infections of the kidney and the biliary passages.

REFERENCES.

Bechhold and Ehrlich, 'Zeitschr. Physiol. Chemie,' vol. 47, p. 173 (1906); *ibid.*, vol. 52, p. 77 (1907).

Benda, 'Ber. Deutsch. Chem. Ges.,' vol. 45, p. 1787 (1912).

Browning and Gilmour, 'Journ. Path. and Bact.,' vol. 18, p. 144 (1913).

Browning, Gulbransen, Kennaway and Thornton, 'Brit. Med. Journ.,' January 20 and July 21, 1917.

Chick, 'Journal of Hygiene, vol. 12, p. 414 (1912).

Morgenroth and Levy, 'Berlin. Klin. Woch.,' 1911, Nos. 33 and 44.

Wright and Windsor, 'Journal of Hygiene,' vol. 11, p. 385 (1902).

* We are indebted to Captain T. F. Cotton, C.A.M.C., for the clinical material and to Dr. S. Russ for the estimations from absorption spectra. It is important that specimens of this substance to be employed for internal administration should be free from admixture with traces of poisonous metals.